Phycobiliprotein fusion proteins: versatile, intensely fluorescent constructs

Alexander N. Glazer*[a], Yuping A. Cai[b], Aaron J. Tooley[c]
[a]Department of Molecular and Cell Biology, 142 LSA, University of California, Berkeley CA USA 94720-3200; [b]Lilly Corporate Center, Indianapolis, IN USA 46285; [c]Department of Pathology, University of California at San Francisco, San Francisco, CA USA 94143-0511

ABSTRACT

Since 1982, phycobiliproteins have served as fluorescent labels in a wide variety of cell and molecule analyses. The exceptional spectroscopic properties of these labels include very high absorbance coefficients and quantum yields, and large Stokes shifts. The spectroscopic diversity of these reagents is restricted to a subset of naturally occurring phycobiliproteins with stable assembly states *in vitro,* whose target specificity is generated by chemical conjugation to proteins or small molecules. The latter step generates heterogeneity. These limitations have been overcome by expressing various recombinant phycobiliprotein constructs in the cyanobacterium *Anabaena* sp. PCC7120. Modular recombinant phycobiliprotein-based labels were constructed with some or all of the following features (a) an affinity purification tag; (b) a stable oligomerization domain (to maintain stable higher order assemblies of the phycobiliprotein monomers at very low protein concentration); (c) a biospecific recognition domain. Such phycobiliprotein constructs are readily purified from crude cell extracts by affinity chromatography and used directly as fluorescent labels. To generate constructs for intracellular *in vivo* labeling, the entire pathways for the biosynthesis of the His-tagged holo-α (phycocyanobilin-bearing) subunit of phycocyanin (emission max. 641 nm) and of the His-tagged holo-α (phycobiliviolin-bearing) subunit of phycoerythrocyanin (emission max. 582 nm) were reconstituted in *Escherichia coli*.

Keywords: Fluorescent labels, recombinant phycobiliprotein, His tag, Strep tag, oligomerization domain, biotinylation domain, bilin biosynthesis

1. INTRODUCTION

Since their introduction in 1982 by Oi, Glazer and Stryer[1] as fluorescent tags, the phycobiliproteins have continued to be exceptionally valuable in many fields of biology, such as cell sorting and cell analyses, histochemistry, and high-density array screening[2].

The natural function of phycobiliproteins is to serve as light-harvesting components of the photosynthetic apparatus of both prokaryotes (cyanobacteria, prochlorophytes) and eukaryotes (red algae, cryptomonads). In cyanobacteria and red algae the phycobiliproteins form macromolecular complexes called phycobilisomes. The *in vivo* assembly states of the phycobiliproteins in cryptomonads and prochlorophytes remain to be determined. The phycobiliproteins carry various linear tetrapyrrole prosthetic groups thioether-linked to specific cysteinyl residues. These prosthetic groups are called bilins because of their close structural relationship to the bile pigments, biliverdin and bilirubin. Indeed, the bilins are derived biosynthetically from biliverdin[3,4].

The spectroscopic properties of a phycobiliprotein are determined by the nature of the bilin(s) it carries and its assembly state[5]. The native phycobiliproteins exist as hexamers, $(\alpha\beta)_6$ or trimers, $(\alpha\beta)_3$. Their molar extinction coefficients and fluorescence quantum yields decrease sharply upon dissociation to the αβ monomer. In studies described here, we have have employed protein engineering to generate in a cyanobacterium recombinant phycobiliprotein constructs that do not dissociate even at extremely low protein concentrations. In addition to a phycobiliprotein subunit, such constructs include modules such as a "leucine zipper" oligomerization domain, a module that includes an affinity tag, and module

*glazer@uclink4.berkeley.edu; phone 510-987-0143

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that serves as biospecific recognition domain. We have demonstrated that such constructs can be readily isolated from crude cell extracts by affinity chromatography and used directly as fluorescent tags with no further manipulation. These engineered tags represent a "second generation" of phycobiliprotein-based fluorescent labels[6].

To address intracellular expression of fusion proteins that include a phycobiliprotein tag, we have built on our earlier work on phycobiliprotein bilin lyases[7,8] and on the recent work of Lagarias and his colleagues on ferredoxin-dependent bilin reductases[4]. To generate constructs for intracellular *in vivo* labeling, we reconstituted in *E. coli* the entire pathways for the biosynthesis of the His-tagged holo-α (phycocyanobilin-bearing) subunit of phycocyanin (emission max. 641 nm) and of the His-tagged holo-α (phycobiliviolin-bearing) subunit of phycoerythrocyanin (emission max. 582 nm).

2. METHODOLOGY AND RESULTS

The methods and results described here are detailed in studies we have published elsewhere. The construction and properties of modular recombinant phycocyanins are described in ref. 6. The engineering of *E.coli* strains able to convert endogenous heme to phycocyanin α subunit-linked phycocyanobilin or phycoerythrocyanin α subunit-linked phycobiliviolin is described in refs. 9 and 10.

In brief, a family of specific cloning vectors was constructed to express in the cyanobacterium *Anabaena* sp. PCC7120 recombinant C-phycocyanin subunits with one or more of the following modules: an affinity tag (the 6xHis tag, abbreviated HT below), a module encoding a GCN4-pII (abbreviated as pII below) or GCN4-pLI oligomerization domain, and a biospecific recognition domain. Examples of the latter were the streptavidin-binding Strep2 tag or the C-terminal 114-residue portion of the *Anabaena* sp. PCC7120 biotin carboxyl carrier protein ($BCCP^{114}$). His-tagged phycocyanin β subunit constructs containing $BCCP^{114}$ at its C-terminus, expressed in *Anabaena* sp. were more than 30% biotinylated. The various tagged α or β subunits of C-phycocyanin formed stoichiometric complexes *in vivo* with appropriate wild type subunits to give constructs with the appropriate oligomerization state and normal posttranslational modifications and with spectroscopic properties very similar to those of unmodified phycocyanin. Over 75% of a protein with the composition $(\alpha\text{:HT-pII-}\beta\text{-}BCCP^{114})_3$, was specifically bound to streptavidin- or avidin-coated beads[6].

We have engineered *E. coli* strains that have the entire pathways for the conversion of endogenous heme to phycocyanobilin, the prosthetic group of the holo-α subunit of phycocyanin or to phycobiliviolin, the prosthetic group of the holo-α subunit of phycoerythocyanin and for the attachment of these bilins to the cognate apo-subunit. The cyanobacterial enzymes required for the conversion of heme to the natural chromophore 3Z-phycocyanobilin, heme oxygenase 1 and 3Z-phycocyanobilin:ferredoxin oxidoreductase, were expressed from a plasmid under the control of the hybrid *trp-lac* (*trc*) promoter. Genes for the apo-subunit (C-phycocyanin α subunit) and the heterodimeric lyase (*cpc E* and *cpcF*) that catalyzes phycocyanobilin attachment[7] were expressed from the *trc* promoter on a second plasmid. Upon induction, the recombinant *E.coli* produced phycocyanin holo-α subunit with spectroscopic properties qualitatively and quantitatively similar to those of the native protein produced in cyanobacteria. About one-third of the apo-subunit was converted to the holo-subunit[9]. The same approach was used to engineer an *E. coli* strain that produced phycobiliviolin-bearing His-tagged holo-α subunit of phycoerythrocyanin. Here, the second plasmid carried the genes for the phycoerythrocyanin-α subunit and for the appropriate heterodimeric lyase/isomerase (*pecE and pecF*)[10] that catalyzes both the covalent attachment of phycocyanobilin and its concurrent isomerization to phycobiliviolin. The latter strain produced phycoerythrocyanin holo-α subunit with spectroscopic properties similar to those of the native protein produced in cyanobacteria. About two-thirds of the phycoerythrocyanin apo-α subunit was converted to the holo-α subunit.

3. CONCLUSIONS

The methodologies described above provide access to a wider range of phycobiliprotein labels. For more than twenty years, the use of phycobiliproteins as tags was limited to proteins which retain their quaternary structure at extremely low protein concentrations. This criterion is met only by the phycoerythrins and by appropriately chemically crosslinked allophycocyanin[2]. Moreover, the use of a phycobiliprotein as a tag requires chemical attachment to a biospecific recognition element, such as biotin, streptavidin, protein A, an antibody, etc. Such conjugates are generally heterogeneous because of variation in the position of the linkage between the components in the conjugate population.

The protein engineering methods described resolve these difficulties and provide ready access to fluorescent proteins not available in nature. Other applications of such phycobiliproteins fusions are described elsewhere[11].

The *E. coli* strains engineered to express fluorescent holo-phycobiliprotein fusions allow use of such tags for *in vivo* labeling. These constructs should be valuable labels in the context of the *E. coli* genome project [see http://www.genome.wisc.edu/] and of protein expression and fluorescence energy transfer-based detection of *in vivo* protein-protein interactions in other bacteria as well.

REFERENCES

1. V.T. Oi, A.N. Glazer, and L. Stryer "Fluorescent Phycobiliprotein Conjugates for Analyses of Cells and Molecules" *J. Cell Biol.* 93, 981-986 (1982).
2. A.N. Glazer *Chemicals from Microalgae*, pp. 261-280, Taylor & Francis Ltd., London, 1999.
3. S.I. Beale *The Molecular Biology of Cyanobacteria*, pp. 519-558, Kluwer Academic Publishers, Boston. 1994.
4. N. Frankenberg, K. Mukuogawa, T. Kohchi, and J.C. Lagarias "Functional genomic analysis of the Hy2 family of ferredoxin-dependent bilin redutases from oxygenic photosynthetic organisms" *Plant Cell* **13,** 965-972 (2001).
5. A.N. Glazer "Light harvesting by phycobilisomes" *Ann. Rev. Biophys. Biophys, Chem.* **14,** 47-77 (1985).
6. Y.A. Cai, J.T. Murphy, G.J. Wedemayer, and A.N. Glazer "Recombinant phycobiliproteins. Recombinant C-phycocyanins equipped with affinity tags, oligomerization and biospecific recognition domains" *Anal. Biochem.* **290,** 186-204 (2001).
7. C.D. Fairchild and A.N. Glazer "Oligomeric structire, enzyme kinetics, and substrate specificity of the phycocyanin α subunit phycocyanobilin lyase *J. Biol. Chem.* **269,** 8686-8694 (1994).
8. L.J. Jung, C.F. Chan, and A.N. Glazer "Candidate genes for the phycoerythrocyanin α subunit lyase Biochemical analysis of *pecE* and *pecF* interposon mutants" *J. Biol. Chem.* **270,** 12877-12884 (1995).
9. A.J. Tooley, Y.A. Cai, and A.N. Glazer "Biosynthesis of a fluorescent cyanobacterial C-phycocyanin holo-α–subunit in a heterologous host" *Proc. Natl. Acad. Sci. USA* **98,** 10560-10565 (2001).
10. A.J. Tooley and A.N. Glazer "Biosynthesis of the cyanobacterial light-harvesting polypeptide phycoerythrocyanin holo-α subunit in a heterologous host" *J. Bacteriol.***184,** 4666-4671 (2002).
11. A.N. Glazer and Y.A. Cai "Multifunctional recombinant phycobiliprotein-based fluorescent constructs and phycobilisome display" *US Patent No.* 6,649,376, Nov. 18, 2003.